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Published in:
Journal of Molluscan Studies

DOI:
10.1093/mollus/eyu072

Publication date:
2015

Citation for published version (APA):
Iwata, Y., Sakurai, Y., & Shaw, P. (2015). Dimorphic sperm transfer traits for alternative mating tactics in loliginid squid. *Journal of Molluscan Studies*, *81*(1), 147-151. https://doi.org/10.1093/mollus/eyu072

Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2015) **81**: 147–151. doi:10.1093/mollus/eyu072
Advance Access publication date: 6 October 2014

Dimorphic sperm-transfer strategies and alternative mating tactics in loliginid squid

Yoko Iwata[1], Yasunori Sakurai[2] and Paul Shaw[3]

[1]*Atmosphere and Ocean Research Institute, University of Tokyo, Kashiwa, Chiba 277-8564, Japan;*
[2]*Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan; and*
[3]*Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Penglais, Aberystwyth SY23 3DA, UK*

Correspondence: Y. Iwata; e-mail: iwayou@gmail.com

(Received 10 December 2013; accepted 12 August 2014)

ABSTRACT

Animal species usually have a single sperm-storage site in the female body, but females of the squid *Heterololigo bleekeri* possess two distinct sperm-storage sites simultaneously. Use of two sperm-storage sites correlates with alternative male mating behaviours: large consorts guard females and place spermatophores inside the oviduct just before spawning, whereas small 'sneaker' males place spermatophores on the membrane around the female's mouth within the arm crown, where a seminal receptacle is present. Previous work showed that spermatophore and sperm morphology diverge between consort and sneaker males. Here we show novel dichotomous adaptations in the sperm-transfer strategy of males of this squid, associated with the use of two distinct sperm-storage sites on females. The spermatangia ejaculated from spermatophores were clearly dimorphic: all spermatangia ejaculated from spermatophores smaller than 12 mm from small sneaker males were drop-shaped, whereas all spermatangia from spermatophores longer than 12 mm from larger consort males were rope-shaped. In addition, the drop-shaped spermatangia were distinct in having a spine on their base, which might reduce the risk of it being shed from the female's body surface. Our findings suggest that existence of alternative sperm-storage sites, and related biological and environmental factors, lead to the evolution of divergent sperm-transfer strategies.

INTRODUCTION

External fertilization is a common reproductive strategy in aquatic organisms. During external fertilization, dilution of sperm is one of the most important factors affecting fertilization success (Levitan & Petersen, 1995; Levitan, 1998). Therefore, animals might evolve behavioural, morphological and physiological traits relating to sperm transfer to minimize the loss of sperm to the environment. However, it is difficult to demonstrate directly how such traits affect sperm transfer, because each animal species usually has only one sperm-transfer environment.

Male cephalopods produce spermatophores, which are cylindrical capsules containing sperm, and transfer them to the female (Drew, 1919). During mating, spermatophores undergo the 'spermatophoric reaction', a complex evagination process of the spermatophoric tunics and membranes leading to extrusion of the sperm mass (Mann, Martin & Thiersch 1966; Drew, 1919; Marian, 2012a). The spermatangium, the everted spermatophore containing the sperm mass, attaches to the female's body by the mechanical anchorage provided by the ejaculatory apparatus and chemical adhesion by the cement body (Hoving, Lipinski & Videler, 2008; Hoving *et al*., 2009; Marian, 2012a,b).

Females of the squid *Heterololigo bleekeri* Keferstein, 1866 have two distinct sperm-attachment sites that are associated with alternative mating tactics; they thus offer an opportunity to examine the evolution of sperm transfer in differing fertilization environments within a single species (Iwata, Munehara & Sakurai, 2005). The remarkable characteristic of this loliginid squid is that females have two separate sperm-attachment sites: on the ventral buccal membrane near the seminal receptacle and on the oviduct in mantle cavity. Use of the two sperm-attachment sites correlates with alternative mating behaviours adopted by males: large consorts guard/hold females and place spermatophores in the oviduct just before spawning, whereas small 'sneaker' males place spermatophores on the female's buccal membrane, near where she holds eggs before depositing them on the spawning substrate (Iwata *et al*., 2005). Furthermore, unlike the other loliginid squid in which consort males attach spermatophores on the outside wall of the oviduct or on the mantle wall near the opening of the oviduct, consort males in *H. bleekeri* attach spermatophores on the inside wall of the oviduct. Fertilization success is far higher for consorts than sneakers in this species (Iwata *et al*., 2005).

The presence of alternative sperm-attachment sites potentially presents two opposing environments for physical and physiological characteristics and sperm competition (e.g. sperm storage and release process, timing to meet eggs) and this should drive males to adopt divergent strategies catering to the conditions at each site. Consistent with this prediction, spermatophore size dimorphism has been found; consort males produce long spermatophores with a rope-shaped spermatangium and sneaker males

produce short spermatophores with a drop-shaped spermatangium (Iwata & Sakurai, 2007; Fig. 1). Sperm-competition theory predicts that sneaker males will produce relatively more sperm than do consorts to compensate for the positional mating advantage of consorts (Parker, 1990; Simmons, Tomkins & Hunt, 1999). Contrary to this expectation, consorts in *H. bleekeri* packaged five-fold more spermatozoa in a single spermatophore than did sneakers (Iwata *et al.*, 2011). Furthermore, two types of fertilization-competent spermatozoa were found in this species (Iwata *et al.*, 2011; Hirohashi *et al.*, 2013). Sneaker males produce 50% longer sperm than do consort males that transfer sperm into internal female body (Iwata *et al.*, 2011). Not only the number but the quality of sperm can be a target for selection through sperm competition, but swimming velocity of sperm did not differ between sneakers and consorts (Iwata *et al.*, 2011). These results are contrary to expectations under simple sperm-competition theory and suggest that the fertilization environment (the other major difference between sneaker and consort strategies) may affect traits of sperm in this species. In addition, only sneaker sperm exhibit chemotaxis towards sperm-emitted respiratory CO_2 (Hirohashi *et al.*, 2013; Hirohashi & Iwata, 2013). These morphological and functional diversifications, which are linked to alternative male mating strategies, could also be related to sperm-attachment sites. Here we investigate further adaptations of the sperm traits that could relate to constraints imposed by distinct sperm-attachment sites.

MATERIAL AND METHODS

Specimens of *Heterololigo bleekeri* were collected from inshore set nets in Aomori, Japan, during the spawning season (January–April in 2007), kept on ice and dissected within 2 d of capture. We sampled 620 individuals (354 males and 266 females) from commercially fished squid that were separated into three size classes: large (less than 18 individuals per 3 kg), medium (18–28 individuals per 3 kg) and small (more than 28 individuals per 3 kg). To cover the whole range of mature adult mantle lengths (MLs, the standard measure of body size in cephalopods) our samples included 36 kg of squid from the large size class (163 individuals: 151 males of 232–399 mm ML, 12 females of 237–304 mm ML), 24 kg from the medium size class (185 individuals: 74 males of 174–324 mm ML, 111 females of 187–254 mm ML) and 24 kg from the small size class (272 individuals: 129 males of 144–304 mm ML, 143 females of 116–276 mm ML). All but five males were fully mature with developed spermatophores. Five immature individuals without developed spermatophores and 21 individuals having only broken spermatophores in their spermatophore storage organ (Needham's sac) were excluded from the analysis.

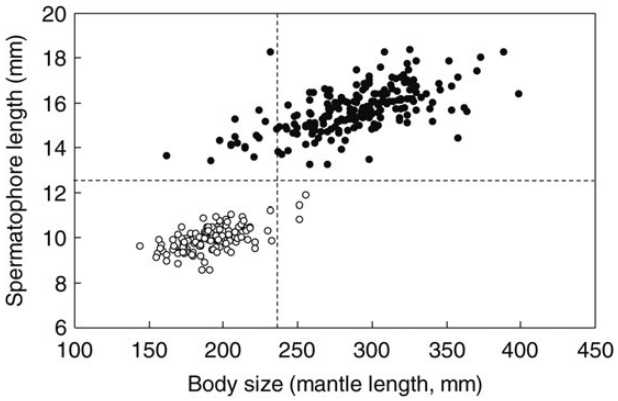

Figure 1. Spermatophore dimorphism associated with two sperm-storage sites in the squid *Heterololigo bleekeri*. The relationship between male mantle length and spermatophore length was similar to that given by Iwata & Sakurai (2007), although it was recalculated with data newly collected in 2007 ($n = 328$). Dashed vertical line shows morphological switch point detected by the model of Eberhard & Gutiérrez (1991) and dashed horizontal line that detected by the model of Kotiaho & Tomkins (2001). Symbols show the morphology of ejaculated spermatangium (open circle, drop-shaped type; solid circle, rope-shaped type).

For each male ($n = 328$), ML and the lengths of 10 spermatophores randomly selected from the spermatophore storage organ were measured with electronic slide callipers. Mean spermatophore length (SL) was calculated for each male. To induce the spermatophoric reaction (ejaculation of spermatangia from the outer spermatophore tunic), spermatophores were removed from the Needham's sac and placed on a Petri dish filled with filtered seawater. The morphology of spermatangia was examined under a stereomicroscope. Males of this species occur in one of two phenotypes, identified by a threshold switch point in the allometric relationship between SL and ML: large males with long spermatophores and small males with short spermatophores. The dimorphism is associated with use of the two alternative sperm-storage sites on the female (Iwata & Sakurai, 2007). According to the previous study, we classified the mature males into the two phenotypes using two partial regression models (Eberhard & Gutiérrez, 1991; Kotiaho & Tomkins, 2001).

For each female ($n = 266$), the two sites of spermatophore attachment (the oviduct opening and the ventral buccal membrane around seminal receptacle) were examined for the presence and morphology of attached spermatangia. Statistical analyses were performed with the program R v. 2.7.

RESULTS

The allometric relationship between ML and SL displayed a clear switch at ML = 236 mm, SL = 12.3 mm: most males larger than ML 236 mm (consorts) had spermatophores substantially longer than 12.3 mm (Fig. 1). The relationship with the model described by Eberhard & Gutiérrez (1991) was $SL = 2.14 + 0.043ML - 0.022(ML - 236)D + 2.16D$ ($D = 0$ when $ML < 236$, $D = 1$ when $236 < ML$, $R^2 = 0.999$, $n = 328$; Fig. 1) and with the model described by Kotiaho & Tomkins (2001) $ML = -16.84 + 21.00SL + 2.08(ML - 12.34)D - 29.96D$ ($D = 0$ when $SL < 12.34$) $D = 1$ when $12.34 < SL$, $R^2 = 0.806$, $n = 328$; Fig. 1).

The spermatangia ejaculated from spermatophores were clearly dimorphic: all spermatangia ejaculated from spermatophores smaller than the switch point ($SL < 12.3$ mm, $n = 124$) were drop-shaped (Fig. 2), whereas all spermatangia from spermatophores longer than the switch point ($SL > 12.3$ mm, $n = 204$) were rope-shaped (Fig. 2). Of the 266 females sampled, 56 had spermatangia attached on the buccal membrane around the seminal receptacle; of these, in 54 only drop-shaped spermatangia were present; two also had rope-shaped spermatangia. In contrast, 159 females had spermatangia attached inside the oviduct, all of which were of the rope-shaped type. All spermatangia attached on both sperm-attachment sites included sperm inside. Putting these results from male and female samples together, we defined males producing spermatophores shorter than 12.3 mm and drop-like spermatangia as sneaker males attaching spermatophores on the buccal membrane. According to this definition, 204 males were classified as consorts and 124 as sneakers.

Drop-shaped and rope-shaped spermatangia also showed two further differences. First, a spine-shaped oral extremity was present on 84.7% of drop-shaped spermatangia, but on only 9.3% of rope-shapede spermatangia (Fig. 2). Logistic regression analysis, with the presence of a spine as the dependent variable and ML or SL as covariates, indicated a significant relationship

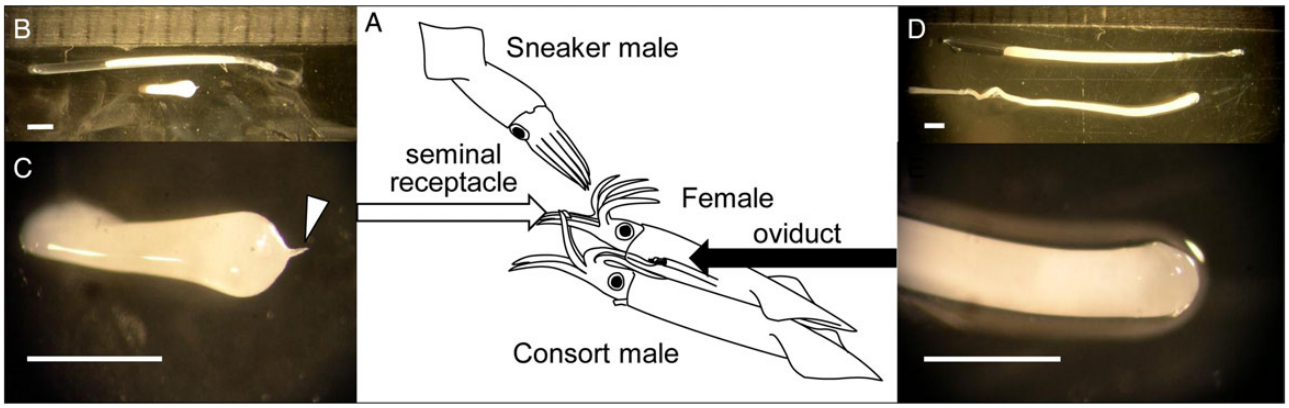

Figure 2. Typical morphology of spermatophores and ejaculated spermatangia of *Heterololigo bleekeri*. **A.** Alternative mating behaviours and the corresponding two sperm-attachment sites. **B.** Short spermatophore and drop-shaped spermatangium with a spine of sneaker male. **C.** Enlarged image of drop-shaped spermatangium. White arrowhead indicates spine on head of spermatangium. **D.** Long spermatophore and rope-shaped spermatangium, without spine, of consort male. **E.** Enlarged image of the oral part of rope-shaped spermatangium. Scale bar = 1 mm.

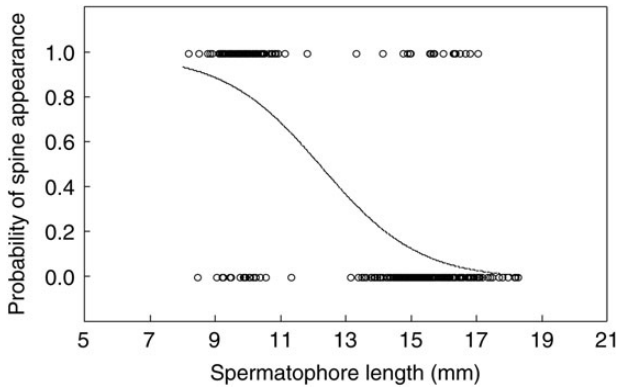

Figure 3. The relationship between spermatophore length and probability of spine occurrence in *Heterololigo bleekeri* ($n = 328$). The line shows the estimated logistic regression curve.

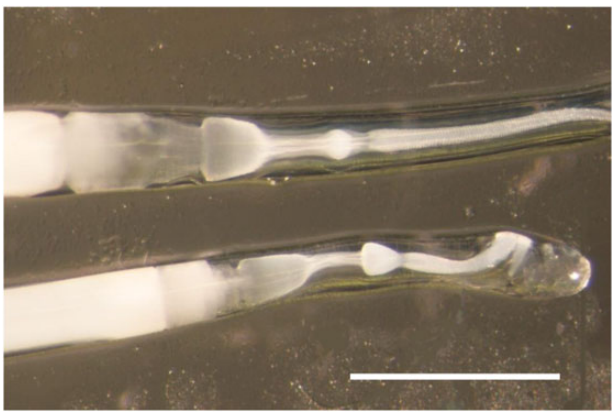

Figure 4. Cement body of intact spermatophores of *Heterololigo bleekeri*. Fresh spermatophores were taken from Needham's sac and put in a Petri dish without being put into water. Oral extremity of the cement body is more developed in short spermatophores of sneaker males (below) than in long spermatophores of consort males (above). Spermatophore of consort male has longer ejaculatory apparatus and the oral tip of spermatophore is out of the frame. Scale bar = 1 mm.

between spine presence and SL, although not with ML (whole model: $\chi^2_2 = 190.75$, $P < 0.001$; SL: $z = -5.989$, $P < 0.001$; ML: $z = 1.145$, $P = 0.252$; Fig. 3). Second, observation of intact spermatophores suggested that the oral extremity of the cement body was better developed in short than in long spermatophores (Fig. 4).

DISCUSSION

In the present study we show that sneaker males produced spermatophores with a well-developed oral extremity of the cement body and with a spine-like structure on the spermatangia, features that were lacking in most consort spermatophores in *Heterololigo bleekeri*. A clear relationship exists between spermatophore morphology and male body size, but the range of male body sizes overlap between sneaker and consort males (Iwata & Sakurai, 2007; Fig. 1). Logistic regression analysis with SL or ML as a covariate suggested a tight link between the presence of a well-developed oral extremity and spermatophore morphology and, therefore, with alternative sperm-storage sites. The spine-like oral extremity of the cement body is likely to be an adaptation to attach the spermatangia to the female buccal membrane and keep it there.

Marine organisms with external fertilization must face a high probability that their gametes will be rapidly diluted. The most important factors for successful fertilization are synchrony of sperm and egg release, proximity of individuals and low water movement (Levitan & Petersen, 1995; Levitan, 1998). Species that broadcast spawn often synchronize gamete release with other individuals using environmental cues, such as lunar cycle (Serrão *et al.*, 1996), depletion of dissolved inorganic carbon (Pearson, Serrão & Brawley, 1998) and weather or hydrodynamic conditions (Borndt, Callow & Brawley, 2002). On the other hand, many species of invertebrates, including cephalopods, have evolved specialized sperm-transfer strategies, e.g. males transfer sperm package within spermatophores to females, which store them until spawning. Females of many cephalopod species have evolved sperm-storage sites (seminal receptacles). In some deep-sea squid species, females lack seminal receptacles and males implant spermatangia directly into the skin of the females (Hoving *et al.*, 2004; Hoving & Laptikhovsky, 2007; Hoving *et al.*, 2008, 2009, 2010; Bolstad & Hoving, 2011).

In the mating system of *H. bleekeri*, sperm of large consort males and small sneaker males experience different storage and fertilization environments, due to both the synchrony of gamete release and hydrodynamic condition. The drop shape of the

sneaker male's spermatangium decreases surface area relative to volume, and might reduce the effect of water flow and thus the risk of loss from the female's body. Unfortunately we were unable to measure water flow between the arms and in the mantle cavity of living females. Both sperm-attachment sites experience some water flow, but are relatively protected compared with the other parts of the female's body surface. The term 'confined external fertilization' has been proposed to describe the situation in which fertilization is external but contained within a confined volume formed by the animal, such as within the mantle cavity or the space within the arms (Naud & Havenhand, 2006). Because consort males of *H. bleekeri* pass spermatophores to the inside wall of the oviduct, their spermatangia must experience far less turbulence than they would elsewhere in the mantle cavity where water is continuously circulated. However it is unclear which sperm-attachment site has higher risk of spermatangium loss and/or sperm dilution. Another important difference for consort and sneaker sperm is the presence of the seminal receptacle on the buccal membrane. Some histological studies of the squid seminal receptacle suggest that released sperm reach the seminal receptacle by active swimming (Drew, 1919; Sato *et al*., 2010). The distal end of the drop-shaped sneaker spermatangia (with the opening from which sperm are released) is closer to the female surface due to its short length. Considering this, and the fact that in *H. bleekeri* only sneaker sperm show chemotaxis toward respiratory CO_2 (Hirohashi *et al*., 2013; Hirohashi & Iwata, 2013), spermatangium morphology may be adaptive in allowing its proximity to the female body surface and in bringing sperm into the seminal receptacle.

Marian (2012a) analysed the details of the spermatophoric reaction and the morphology of the spermatophore and spermatangium in *Doryteuthis plei*, which has similar alternative mating tactics to *H. bleekeri* (Hanlon & Messenger, 1996). In that study, the oral extremity of the cement body of the spermatangium had a complex structure with some crests. Although the observations of spermatangium morphology were conducted only for consort spermatophores, histological observation of implanted spermatangia on the female body showed that both consort and sneaker spermatangia had the characteristic structure of the oral extremity of the cement body and that both were deeply thrust into the female tissue (Marian, 2012a). An arrowhead structure has been found in a similar location in spermatangia of the squid *Todarodes pacificus*, a species that uses only one mating behaviour, transferring spermatophores to the buccal membrane of females (Takahama & Kinoshita, 1991). In *T. pacificus*, the spermatangium is drop-shaped and has a clear arrowhead protuberance on the head. This arrowhead structure has also been observed in the intact spermatophores (Takahama & Kinoshita, 1991). Takahama & Kinoshita (1991) hypothesized that the arrowhead protuberance should function in adherence to the female body surface. Mating behaviour of sneaker males in loliginid squid is similar to the mating behaviour of *T. pacificus*; in both, males pass spermatophores to the female buccal membrane. These studies support the hypothesis that the oral extremity of the cement body in cephalopod spermatangium is an adaptation to implant the structure into the female body surface. However, the functional significance remains elusive, as some studies have shown that the oral extremity of the cement body is not necessary to attach the spermatangia to the female body. In some deep-sea species, notably *Rossia moelleri* (Hoving *et al*., 2009), two onychoteuthid species (Bolstad & Hoving, 2011) and *Architeuthis* sp. (Hoving *et al*., 2004), spermatangia are implanted into female tissue without a specific structure on the oral end of the spermatangium. Recent studies have shown that the ejaculatory apparatus of the spermatophore has numerous stellate particles and that it plays an important role in attaching the spermatangium to female tissue (Marian, 2011, 2012a, 2012b; Marian *et al*., 2012). Further study of our findings on the intraspecific dimorphism associated with the alternative sperm-attachment sites could help to understand the function and the evolution of spermatangium morphology in cephalopods.

In conclusion, morphological adaptations involved in sperm-transfer strategies, linked to alternative mating behaviours, are closely associated with distinct sperm-attachment sites. Our findings further support that the fertilization environment is one of the strongest evolutionary forces acting on male reproductive traits.

ACKNOWLEDGEMENTS

We thank A. Gange, V. Jansen, A. Takahashi, Y. Watanuki and R. Yanagimachi for comments and K. Ito for collecting samples. We greatly appreciate two anonymous reviewers and Associate Editor J. Voight helped to improve the manuscript. This study was supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18789001), the European Commission (PIIF-GA-2008-220611) and the Japan Society for the Promotion of Science Fellowship to YI.

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